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TBA_{225}, a fusion toxoid vaccine for protection and broad neutralization of staphylococcal superantigens

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Superantigens (SAgs) play a major role in the pathogenesis of *Staphylococcus aureus* and are associated with several diseases, including food poisoning, bacterial arthritis, and toxic shock syndrome. Monoclonal antibodies to these SAgs, primarily TSST-1, SEB and SEA have been shown to provide protection in animal studies and to reduce clinical severity in bacteremic patients. Here we quantify the pre-existing antibodies against SAgs in many human plasma and IVIG samples and demonstrate that in a major portion of the population these antibody titers are suboptimal and IVIG therapy only incrementally elevates the anti-SAg titers. Our *in vitro* neutralization studies show that a combination of antibodies against SEA, SEB, and TSST-1 can provide broad neutralization of staphylococcal SAgs. We report a single fusion protein (TBA_{225}) consisting of the toxoid versions of TSST-1, SEB and SEA and demonstrate its immunogenicity and protective efficacy in a mouse model of toxic shock. Antibodies raised against this fusion vaccine provide broad neutralization of purified SAgs and culture supernatants of multiple clinically relevant *S. aureus* strains. Our data strongly supports the use of this fusion protein as a component of an anti-virulence based multivalent toxoid vaccine against *S. aureus* disease.

Staphylococcus aureus (SA) is a leading cause of hospital and community-associated infections worldwide with no effective vaccines available[1]. The remarkable ability of SA to cause a wide range of diseases from skin and soft tissue infections (SSTI) to life threatening sepsis and pneumonia is in part due to its ability to escape the immune response using a plethora of virulence factors: the superantigenic and pore-forming toxins, coagulase, capsular polysaccharide, adhesins, proteases, and complement inactivating exoproteins[2]. Since its first emergence in the 1960s methicillin-resistant SA (MRSA) has become endemic in healthcare settings, and more recently also within the community, posing a major global challenge[3,4]. There have hence been increasing efforts directed towards the development of vaccines and therapeutics for SA infections. However, to date, no successful vaccine or antibody against SA infections has been developed, and there has been a spate of clinical trial failures on this front[1,5–8].

Targeting SA toxins represent an alternative approach as "anti-virulence" vaccine for prevention of severe *SA* disease. Staphylococcal pore forming toxins alpha and gamma hemolysins and leukotoxins play critical roles in immune evasion, by killing cells of the first line of defense such as neutrophils, monocytes, and macrophages, providing iron for bacterial growth by lysing red blood cells, or enabling dissemination of bacteria through killing of cells with critical barrier function such as epithelial cells[2]. Pyrogenic superantigens (SAgs) represent a major family of SA toxins composed of staphylococcal enterotoxins (SEs) and toxic shock syndrome toxin 1 (TSST-1). In contrast to conventional antigens that undergo proteolytic processing by antigen presenting cells to be presented as MHC/peptide complex to T cells, SAgs cross-link T cell receptor (TCR) with MHC Class II and activate up to 30% of T cells[9] leading to a massive release of cytokines and chemokines, enhanced expression and activation of cell-adhesion molecules, increased T-cell proliferation, and eventually T cell apoptosis or anergy. This sequence of events can culminate in toxic shock syndrome (TSS), a life-threatening condition characterized by rash, hypotension, fever, and multisystem dysfunction[10]. Antibodies play an important role in protection against TSS, thus individuals that do not seroconvert towards the offending toxin due to hypo responsive T-cells[11] and/or T-cell dependent B-cell apoptosis[12] are more likely to experience recurring bouts. Furthermore, SAgs impact the virulence of SA strains through induction of a local excessive inflammatory response, immune subversion by

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inducing apoptosis of T and B cells[11,12], modulating the function of regulatory T cells (Tregs)[13–15], innate lymphoid cells (ILCs)[16] and unconventional T cells such as γδ T cells[17,18], NKT cells[19–21], and mucosa associated invariant T (MAIT) cells[22]. Besides TSS, SAgs along with other *S. aureus* toxins contribute to pneumonia, infective endocarditis, neonatal exanthematous disease, sepsis, and atopic dermatitis[23–25].

A major challenge for development of vaccines against SAgs is the fact that various SA strains can produce one or more of over twenty superantigens[26]. While the primary sequence identity among SAgs is limited they exhibit considerable structural homology[27]. Consistent with this structural homology some limited antibody cross-reactivity to certain superantigens has been reported[28]. Most people have been exposed to SA and have antibodies to SAgs and other SA toxins[29]. Intravenous immunoglobulin (IVIG) has been used to treat TSS caused by streptococcal and staphylococcal SAgs, however with limited success[30–33]. Thus, developing broadly cross-neutralizing vaccines and immunotherapeutics for SAgs is highly desirable.

Our present study, based on measurement of anti-SAg antibodies in human plasma and IVIG, suggests that patients can benefit from immunization with SAg-based vaccines that elicit an anamnestic antibody response to three superantigens SEA, SEB, and TSST-1. We demonstrate that antibodies against these SAgs, affinity purified from IVIG, exhibit a wide range of cross-neutralizing activity towards various SAgs with the best activity observed when such antibodies were combined. Based on this finding we generated a fusion protein of rationally designed toxoids for SEA, SEB, and TSST-1. Here, we demonstrate that this fusion vaccine (TBA_{225}) is free of superantigenic toxicity and elicits highly protective antibody responses that cover a wide range of SAgs produced by various clinically relevant SA strains. Thus, this vaccine is a prime candidate for inclusion into a multivalent *S. aureus* vaccine.

Experimental Procedures

Bacterial superantigens and endotoxin. Superantigens SEA, SEB, SEC-1, SEC-2, SEC-3, SED, SEE, SEH, SEK, TSST-1, SpeA and SpeC (certified as >95% purity by SDS) were purchased from Toxin Technology (Sarasota, FL), reconstituted with deionized water and stored at −80 °C until use.

Affinity purification of human anti-SAg antibodies. SEA, SEB and TSST-1 were coupled to agarose beads (1 mg SAg/mL of bead volume) of an Aminolink® plus immobilization column (ThermoScientific, Rockford, IL) following the manufacturer's protocol. Affinity purification of specific antibodies from commercial IVIG (Omrix Biopharmaceuticals, Nes-Ziona, Israel) was carried out according to manufacturer's protocol with minor modifications: 50 mL of IVIG was incubated with toxin-coupled beads for 90 minutes at room temperature (RT) with gentle rocking, centrifuged, and the supernatant removed and a fresh 50 mL of IVIG incubated with the beads for another 1 hour and 30 minutes. Elution was performed with glycine HCl pH 2.5 buffer. Eluted fractions were collected in neutralizing buffer, containing 0.1 M Tris at pH 9 (final pH 6–7). 37.5 µl of affinity-purified anti-SEA, -SEB, - TSST-1, in semi-log dilutions (0.02–20 µg/ml) or IVIG in semi log dilutions (2.5–2500 µg/ml) and 37.5 µl of a 1 ng/ml preparation of either SEB, SEC. 1–3, SEE, SEH, SEI, SEK, TSST-1, SpeC, or 2 ng/ml of SED, or 3 ng/ml of SpeA were mixed. To test the synergistic activity of purified polyclonal Abs a combination of anti-SEA, -SEB, and –TSST-1 were used in a semi log dilution ranging from 0.02 to 20 µg/ml along with the same amount of toxin as above.

Production of fusion constructs. The genes encoding the toxoids were codon optimized, synthesized, cloned into the pET24a (+) expression vector and transformed into BL21(DE3) *E. coli* cells. Overnight cultures were expanded in Luria Broth containing kanamycin until a mid-log phase culture (~0.5 OD at 600 nm), at which point the cells were chilled to ~25 °C and induced with 0.3 mM IPTG, followed by overnight culture at 25 °C. The cells were then harvested, weighed, and resuspended in cell lysis buffer (20 mM Tris pH 8.0, 50 mM NaCl, 1 mM EDTA, 0.1% Triton X-100). Lysozyme was added (1 mg/mL), the cells incubated at 37 °C for 30 minutes, and then the partially lysed cells were sonicated. The cell lysate was adjusted to 0.5 M NaCl, and the nucleic acid was precipitated by the addition of polyethyleneimine (PEI) under constant mixing. The PEI pellet was removed by centrifugation, and the supernatant containing the toxoid was subjected to ammonium sulfate (ASO_4) precipitation. The ASO_4 pellets were recovered by centrifugation and stored at −80 °C. The ASO_4 pellets were resuspended and desalted into the capture column equilibration buffer, clarified, and subjected to chromatography over a Poros 50 HS cation exchange column. The column was equilibrated, loaded, washed and eluted using a 40-column volume (CV) gradient from 25 to 1,000 mM NaCl in phosphate buffer at pH 6.5. The column fractions were analyzed by SDS to determine the toxoid containing fractions, the pooled material dialyzed into the next column equilibration buffer and subjected to chromatography over a BioRad ceramic hydroxyapatite (HTP) (40-micron bead) Type I column. The column was equilibrated, loaded, washed and eluted using a 40 CV gradient of 50–1,000 mM NaCl in a phosphate buffer at pH 6.8. The fractions were analyzed by SDS and the pooled HTP fractions were dialyzed into the appropriate storage buffer, filter sterilized, aliquoted and frozen at −80 °C.

The fusion constructs were then characterized by SDS, WB and HPLC. For WB, primary antibody for the sample (rabbit anti-SEA, SEB, TSST pAb) (0.25 µg/ml) and goat anti-rabbit IgG alkaline phosphatase conjugate (Bio-Rad) secondary antibody (1:3000, v/v) were used. For SEC-HPLC, 10–80 µg of TBA_{225} were injected in an Agilent Technologies 1260 Infinity Series instrument using an AdvanceBio SEC. 300 Å 7.8 × 300 mm LC column with a mobile phase of 50 mM sodium phosphate buffer + 150 mM NaCl, pH 7.0 running at a flow rate of 0.5 mL/min. The chromatogram generated by the Agilent OpenLabs software plots absorbance at 280 nm as a function of retention time. All analysis of the peaks was performed by the auto-integrate function in the OpenLabs software.

Thermal stability. The thermal stability of TBA_{225} and the individual toxoids was determined by differential scanning fluorimetry (DSF) as described previously[34]. Briefly, 5 µL of 10X SYPRO-Orange dye (Invitrogen, Carlsbad, CA, USA) was added to each sample for a final reaction volume of 25 µL. The thermal assay was

conducted in a BioRad CFX Connect thermocycler with the temperature ramped from 30 to 99 °C at intervals of 0.1 °C/6 s. The melting temperature (Tm) for each sample is defined as the vertex of the first derivative (dF/dT) of relative fluorescence unit (RFU) values. Bovine serum albumin (Pierce) with a Tm of 60 °C ± 0.0 was used as a control.

Human cells and plasma samples. Commercially-sourced human peripheral blood mononuclear cells (PBMCs) were collected and isolated, using the Advarra Institutional Review Board (https://www.advarra.com/) with a peer-approved protocol, from heparinized blood of non-study de-identified healthy human donors by Ficoll gradient centrifugation as described elsewhere[35] and stored in liquid nitrogen until further use. Deidentified plasma samples were received from Omrix Biopharmaceuticals (Nes-Ziona, Israel). All studies involving human samples were performed in accordance with the applicable guidelines and regulations.

PBMC stimulation profile of toxoids. 75 µl of PBMC cell suspension (at 1.5×10^5 cells) with a viability of >95% was then added to a 96-well plate containing the toxoid or toxin to be tested diluted semi-log serially starting at 1000 ng/ml. After incubation at 37 °C in 5% CO_2-95% air for 48 hours, the plates were centrifuged at 1500 rpm for 10 minutes, culture supernatants harvested and IFNγ concentration (pg/ml) was determined by ELISA (Human IFN-gamma DuoSet, R&D Systems, Minneapolis, Minn.) following the manufacturers' protocol.

Serology ELISAs. Serology ELISAs were performed as described previously[36]. Briefly, 96-well plates were coated with 100 ng/well of wild type (WT) proteins overnight at 4 °C. After washing, plates were then blocked for one hour at room temperature (RT) followed by three washes. Plates were incubated for one hour at RT with the test serum samples (diluted semi-log) and washed three times before applying goat anti-mouse IgG (H&L)-HRP (Horse Radish Peroxidase) in starting block buffer. Plates were incubated for one hour at RT, washed, and incubated with TMB (3,3′,5,5′-tetramethylbenzidine) to detect HRP activity for 30 min. Optical density at 650 nm was measured using a Versamax™ plate reader (Molecular Devices, CA). Data analysis for full dilution curves was performed using the SoftmaxPro software version 5.4.5 (Molecular Devices, CA).

Quantitative ELISA for anti-SAg antibodies in plasma and IVIG. Standard anti-SAg IgGs were established using affinity purified human antibodies against SEA, SEB, SED, TSST-1, SpeA, and SpeC. The purified antibodies were quantified by BCA assay. Standard curves were established using a full dilution of each antibody on wells of a 96 well plate coated with the respective toxins and bound antibodies detected with HRP-conjugated anti-human IgG. The standard curve (4PL) was repeated 10 times with a CV of <20% with respect to the inflection point and lower and upper asymptotes. To measure the concentration of each anti-SAg antibody content in plasma samples they were run in duplicates and at two dilutions along with the respective standard and the concentration of the anti-SAg in the unknown plasma determined using a 4PL curve fit.

Adsorption studies. SAg cocktail, individual components SEA, STEBVax, TSST-1, TBA and TBA_{225} were incubated with Alhydrogel at various antigen: adjuvant ratios for half hour at RT. After incubation, the antigen-adjuvant mixture was centrifuged, and the supernatant was detected on an SDS gel. Adsorption of antigens to Alhydrogel was indicated by a thin or negligible band of protein visible on the supernatant SDS as compared to control without any Alhydrogel depicted by the non-adsorbed complete antigen band.

Animal studies. Six to eight-week-old female Balb/c mice were purchased from Charles River (Wilmington, MA). All mouse work was conducted in accordance with protocols approved by Integrated BioTherapeutics' institutional animal care and use committee (IACUC) and applicable guidelines and regulations.

For prophylactic protection studies in BALB/c mice, 1.25 µg of superantigen was pre-incubated with 125 µg of total IVIG or purified human polyclonal Abs for 1 h at room temperature before intraperitoneal (IP) administration in a total volume of 200 µl. Four hours post injection, SAg toxicity was potentiated with 40 µg of LPS administered intraperitoneally. To evaluate the therapeutic activity of human polyclonal anti-SEB antibodies mice were challenged with 1.25 µg of SEB at t = 0 h and received 40 µg of LPS at t = 4 h via IP route. At t = 6 h, mice were treated with 125 µg of purified human polyclonal anti-SEB antibodies. Mice were monitored for morbidity (weight loss, hunched posture, lethargy, ruffled fur) and mortality over a period of 4 days.

Generation of rabbit polyclonal sera to TBA_{225}. Anti-TBA_{225} polyclonal was generated (GenScript, Piscataway, NJ 08854, USA) using TBA_{225} protein (>95% purity) as an immunogen. Immunizations were done for four rabbits on day 0, 14, 21 with 0.2 mg protein per rabbit with Freud's Incomplete Adjuvant injected subcutaneously. Test bleeds and production bleeds were performed on day 21 and day 42 (GenScript). Each serum from production bleed was individually characterized for ELISA titer and TNA titers before pooling together. Pooled anti-serum was purified by Protein A affinity chromatography into total IgG and labeled as anti-TBA_{225} polyclonal antibody.

Superantigen neutralization assay. PBMCs were prepared in the same way as described above. 75 µl of this cell suspension (1.5×10^5 cells) with a viability of >95% was then added to a 96-well plate containing 75 µl of antibody/toxin mixed at 1:1 as follows: semi-log dilutions of sera starting at 1:40 or antibody starting at 1000 µg/ml and a 0.1 ng/ml preparation of SEB, 1 ng/ml of either SEA, TSST-1 or SEC-1, 0.3 ng/ml of SpeC, or 3 ng/ml of SED, SEE, SEK or SEH. Wells containing medium with toxin only served as positive controls. The plates were incubated at 37 °C in an atmosphere of 5% CO_2-95% air for 48 hours. Plates were centrifuged at 1500 rpm for 10 minutes, culture supernatants harvested and IFNγ concentration (pg/ml) was determined by ELISA. Plates were read at 450 nm using the Versamax plate reader and data was analyzed in Excel.

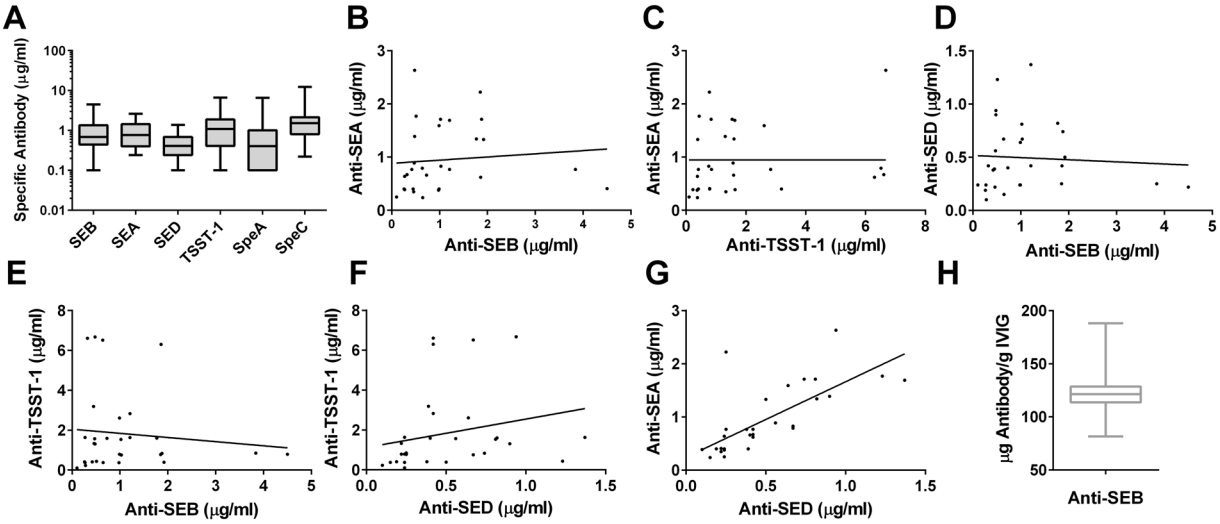

Figure 1. Presence of anti-superantigen antibodies in human plasma and commercial IVIG. (**A**) Range of antibodies against staphylococcal (SEB, SEA, SED TSST-1) and streptococcal (SpeA and SpeC) superantigens in human plasma (n = 30). (**B**–**G**) Correlation of antibody titers to staphylococcal superantigens in human plasma (**H**) Median concentration of anti-SEB polyclonal antibodies in commercially available human IVIG (n = 28).

Preparation of bacterial supernatants and neutralization using TBA_{225} polyclonal. Overnight grown bacterial culture supernatants in tryptic soy broth (TSB) were normalized based on culture OD at 600 nm and sterile filtered through 0.2 μm filter. Bacterial supernatants were then diluted semi-log fold in the interferon-gamma production assay previously described. Dilutions of the supernatants at which ~1000–3000 pg/ml interferon-gamma response was produced were selected and then tested for neutralization by TBA_{225} rabbit polyclonal antibody as well as by $LukF_{mut1}$ rabbit polyclonal antibody (negative control).

Results

Anti-SAg antibodies in human plasma and IVIG. Given the ubiquity of *S. aureus* strains most humans likely have a memory response to staphylococcal antigens such as SAgs due to environmental exposure. A SAg vaccine will likely boost this memory response in an anamnestic fashion. While several groups have reported anti-SAg neutralizing antibodies in IVIG and human serum[37–41], little is known about the exact quantity of anti-SAg antibodies and their cross-neutralizing profile. Using a quantitative ELISAs to measure human antibodies against four staphylococcal SAgs SEA, SEB, TSST-1, and SED as well as two streptococcal SAgs: streptococcal pyogenic exotoxins A (SpeA) and C (SpeC), we quantified the content of antibodies against these toxins in 30 plasma samples from healthy individuals. As shown in Fig. 1A, the range of plasma antibodies against these toxins was comparable and in the range of 0.1–4.5 (Median 0.7) μg/ml for SEB, 0.24–2.6 (Median 0.77) μg/ml for SEA, 0.1–1.4 (Median 0.4) μg/ml for SED, 0.1–6.7 (Median 1.1) μg/ml for TSST-1, 0.1–6.6 (Median 0.41) μg/ml for SpeA, and 0.22–12.4 (Median 1.5) μg/ml for SpeC. The antibody concentrations against various SAgs generally did not strongly correlate with each other (Fig. 1B–F) suggesting independent exposures to strains expressing various SAgs. An exception was a strong correlation between anti-SEA and anti-SED IgG concentrations (Fig. 1G) suggesting co-exposure or strong cross reactivity. These data suggest that, while there is a wide range of anti-SAg response in individuals as previously reported[37–41], the level of cross reactivity of the naturally elicited antibodies is fairly limited and may require vaccination.

Human IVIG is used as adjunctive therapy to treat toxic shock induced by staphylococcal and streptococcal superantigens (SAgs) with limited success[30–33]. This may relate to insufficient level of anti-toxin antibodies in IVIG. We evaluated the concentration of anti-SEB IgG in 28 lots of commercial IVIG. The anti-SEB concentrations ranged from 4.1.1 to 9.4 μg/ml (Median 6.1 μg/ml) corresponding to 82–188 μg per gram IVIG (Median 121 μg/g) (Fig. 1H).

To further evaluate the potency of these natural anti-SAg antibodies we enriched polyclonal antibodies (pAbs) against SEA, SEB, and TSST-1 in IVIG (pooled from 28 lots) by affinity purification. The neutralizing activity of the preparations toward the respective toxins was then tested in PBMCs using IFNγ release as indication of superantigenicity. As shown in Fig. 2A, purified anti-SEA, anti-TSST-1, and anti-SEB pAbs exhibited approximately 1200-fold, 200-fold, and 900-fold enrichment of neutralizing activity compared to IVIG, respectively. With 50% inhibitory concentration (IC_{50}) values in the range of 15–20 ng/ml (~0.5–0.6 nM) the specific antibodies appear to have very strong neutralization capacities. The purified antibodies were then tested for protection in toxin challenge animal models.

Human polyclonal antibodies provide protection *in vivo* against toxin challenge. We tested the protective efficacy of purified human anti-SAg antibodies over a period of 5 days *in vivo* in the LPS potentiation model of toxic shock as previously described[42]. Approximately 1.25 μg/mouse of SEA, TSST-1 or SEB was either mixed with 125 μg of their homologous purified antibody or with 125 μg of IVIG (molar ratio: ~20 Ab:

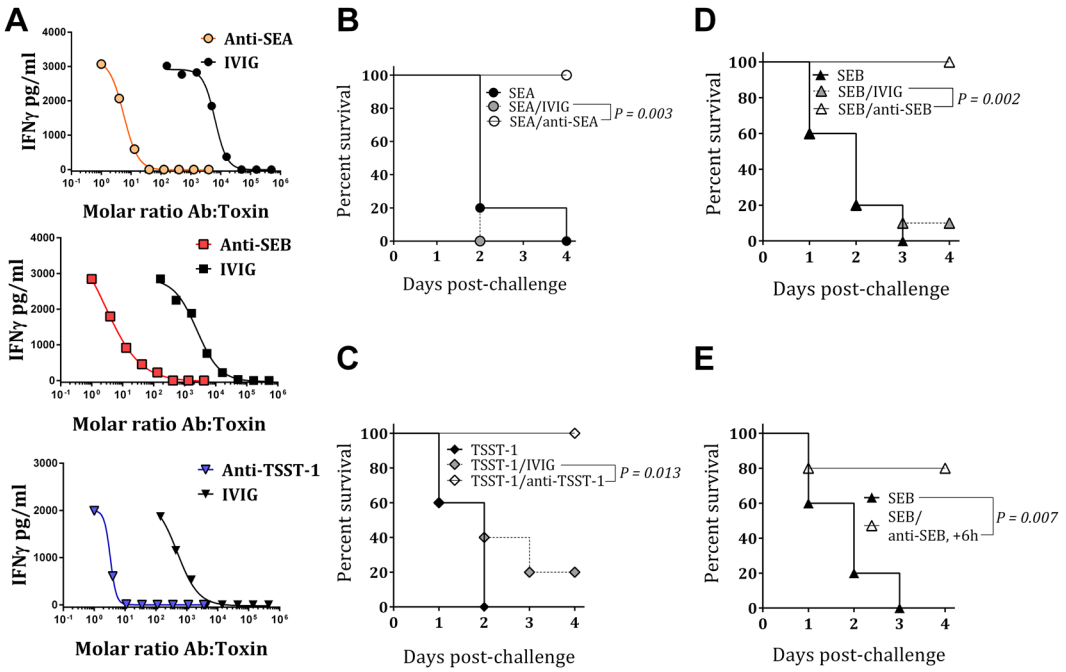

Figure 2. *In-vitro* and *in-vivo* neutralizing activity of purified human polyclonal antibodies. (**A**) Increased toxin neutralizing activity of purified human polyclonal antibodies compared to IVIG. (**B**–**D**) Active protection: mice were challenged with toxins either pre-incubated with purified human polyclonal Abs (open symbols) or IVIG (grey symbols) or with toxin only (black symbols). (**E**) Passive protection: mice were treated with anti-SEB pAbs 6 h post SEB challenge.

toxin) and incubated for 1 hour at RT before IP administration into Balb/c mice. After 4 h mice were challenged with 40 µg LPS. As shown in Fig. 2B–D, mice challenged with the combination of each toxin and its homologous affinity purified pAb (SEA/anti-SEA, SEB/anti-SEB or TSST/anti TSST-1) were 100% protected. In contrast, pre-incubation of IVIG and the toxin resulted in 0% survival for SEA (Fig. 2B), 20% survival for TSST-1 (Fig. 2C), and 10% survival for SEB (Fig. 2D). None of the control mice that received toxin alone survived the challenge.

To test a potential therapeutic effect of polyclonal antibodies, mice were challenged first with 10 LD_{50} of SEB followed by 40 µg LPS, and then treated with 125 µg of purified human anti-SEB pAbs 6 hours post SEB challenge. All control mice, receiving no antibody, died within 3 days, whereas 80% of the mice receiving anti-SEB pAbs survived (Fig. 2E). These data suggest that (i) human anti-SAg antibodies can protect against SAg mediated TSS post toxin exposure, and (ii) the concentration of anti-SAg antibodies in IVIG is probably insufficient to provide meaningful protection in this TSS model.

Broad neutralization towards homologous and heterologous SAgs with antibodies to SEA, SEB and TSST-1. A major challenge for targeting SAgs by vaccines and immunotherapeutics is the fact that various *S. aureus* strains can produce more than 20 different SAgs. Despite low sequence identity in the primary structure, SAgs have highly conserved three-dimensional structure[43] and cross-reactivity between anti-SAg antibodies has been reported[44]. Since SEA, SEB, and TSST-1 represent a divergent set of SAgs, we investigated if a combination of anti-SEA, -SEB and -TSST-1 pAbs could neutralize a broad spectrum of SAgs. We evaluated the neutralizing activity of individual affinity purified human anti-SEA, -SEB and -TSST-1 pAbs, or a cocktail of the three antibodies (from here on referred to as cocktail) towards a wide range of staphylococcal SAgs as well as the related streptococcal SpeA in toxin neutralization assays using PBMCs from five healthy donors. IFNγ production in PBMC culture was used as a readout and the IC_{50} values were determined for each stimulating toxin. Figure 3 shows the molar ratio at IC_{50} over toxin concentration as a measure of the relative homologous and heterologous neutralizing potency of each of the purified pAbs, the cocktail, and IVIG (mean ratio and SD of five donors). As expected, anti-SEA, anti-SEB, and anti-TSST-1 pAbs displayed the highest activity (lowest Ab:toxin ratio) against the homologous SAg with the respective IC_{50}: toxin ratio being about 10–30. All affinity purified antibodies exhibited a higher potency toward heterologous toxins as compared with IVIG (black bars in Fig. 3 showing the highest ratios). Anti-SEA pAbs (orange bars, Fig. 3) exhibited strong cross neutralization of SEE and SEH with ratios of 7, and 30 respectively. Anti-SEB (green bars) cross neutralizing activity was evident towards SEC. 1–3, and to a lesser extent against SEE, SEH, and SpeA. TSST-1 (blue bars) displayed the lowest level of cross neutralization. However, the cocktail (red bars) was cross-neutralizing towards all superantigens at levels equivalent or better than individual antibodies. The lowest level of neutralization by the cocktail was observed against SED and SEK with mean ratios of ~300 and 130 respectively (IC_{50} values: 4.4 and 0.72 µg/ml respectively). However, these levels still represented ~127 and 57-fold increased neutralizing potency, respectively, as compared to IVIG.

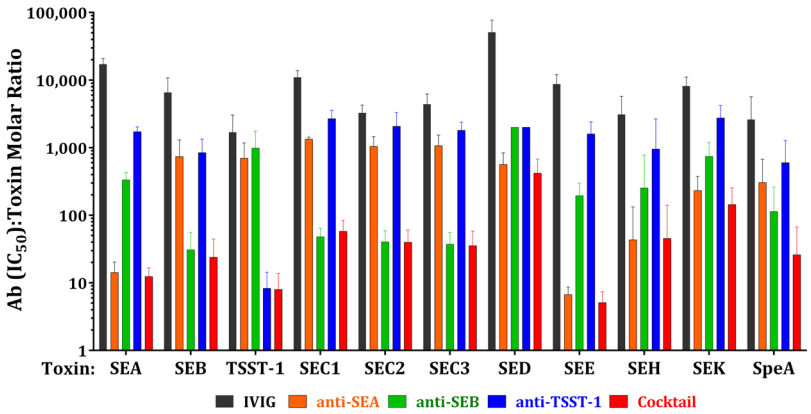

Figure 3. Broad neutralization of various superantigens. Anti-SEA, - SEB and -TSST-1 pAbs purified from IVIG, IVIG and a cocktail of purified pAbs were tested for neutralizing activity of a wide range of staphylococcal superantigens and streptococcal SpeA.

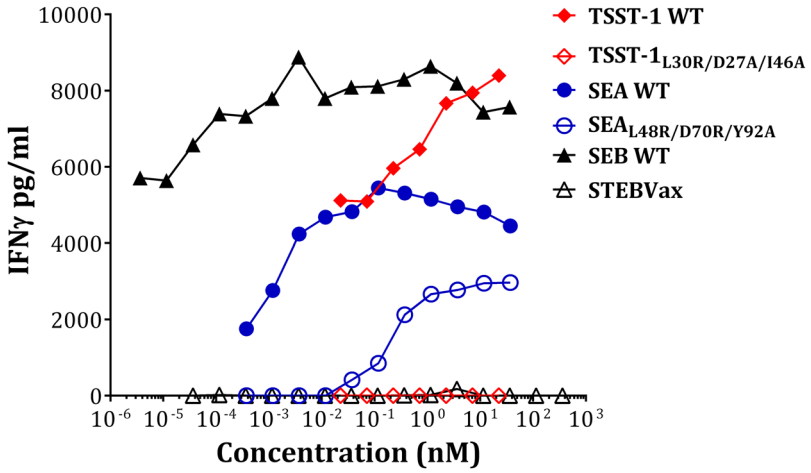

Figure 4. PBMC stimulation (interferon-gamma response) profile of wild-type super antigens TSST-1, SEA, SEB and their respective mutants TSST-$1_{L30R/D27A/I46A}$, $SEA_{L48R/D70R/Y92A}$ and STEBVax.

In summary, these data indicate that broad neutralization of multiple SAgs can be achieved with a combination of potent neutralizing antibodies to SEA, SEB, and TSST-1 suggesting that such responses may be elicited with a multivalent toxoid vaccine against these three toxins.

Generation and characterization of a fusion toxoid vaccine for SEA, SEB, and TSST-1. SAgs cross-link the MHC class II on the surface of antigen presenting cells (APC) with the TCR on the surface of T cells. By engineering three point mutations in the MHC binding surface of SEA, SEB, and TSST-1 inactivated toxoids: $SEA_{L48R/D70R/Y92A}$, $SEB_{L45R/Y89A/Y94A}$ (STEBVax), and TSST-1 -$TSST_{L30R/D27A/I46A}$) were generated and tested in animal models of toxic shock[45–51]. We sought to examine if these three toxoids can be fused into a single polypeptide as a vaccine candidate.

Superantigens are known for their potentially life-threatening toxicity, and hence a superantigen vaccine must be carefully analyzed for its safety. While the safety of STEBVax has been extensively evaluated including a phase I clinical trial that we recently conducted[52], the safety of SEA and TSST-1 toxoids has not been extensively studied. We were also concerned that fusion of the three toxoids may exacerbate some residual superantigenic activity. Thus, we first evaluated the IFNγ response of PBMC from healthy human donors to wildtype SEA, SEB and TSST-1 in comparison to their mutant counterparts. As shown in Fig. 4, TSST-$1_{L30R/D27A/I46A}$ as well as STEBVax showed no IFNγ response at the highest concentrations tested, however, $SEA_{L48R/D70R/Y92A}$, although largely attenuated, exhibited clear IFNγ response at concentrations above 0.1 nM indicating that it retains some residual activity. Previously it was reported that mutation of H225 in the high-affinity MHC binding site of SEA reduces the ability of the toxin to activate T cells[53,54]. Therefore, we introduced H225 A as an additional safety mutation into the SEA-triple mutant to generate $SEA_{L48R/D70R/Y92A/H225A}$.

Two fusion cDNAs were constructed fusing the coding regions for the three mutants for TSST-1, SEB, and SEA, with and without the H225A mutation, named TBA and TBA_{225}, respectively. The individual proteins were spaced with a flexible linker consisting of three GGGGS repeats (3xG4S) (Fig. 5A). The fusion proteins

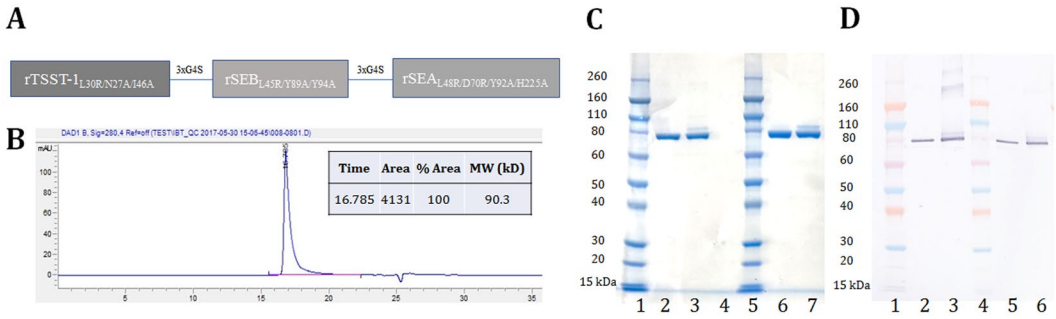

Figure 5. Biophysical characterization of TBA_{225}. (**A**) Sequence of TBA_{225} (**B**) SEC-HPLC of TBA_{225} (**C**) SDS-PAGE for TBA in comparison to TBA_{225}. Lanes 1 and 5: Protein ladder. Lane 2 and Lane 3: 1 µg of reducing and non-reducing versions of TBA. Lane 6 and Lane 7: 1 µg of reducing and non-reducing versions of TBA_{225} respectively. (**D**) Western blot for TBA in comparison to TBA_{225} at reducing as well as non-reducing buffer conditions. Lanes 1 and 4: Protein ladder. Lanes 2 and 3: 25 ng of reducing and non-reducing versions of TBA. Lanes 5 and 6: 25 ng of reducing and non-reducing versions of TBA_{225}.

Protein	Avg Tm (°C) ± Std. Error
TBA	41.8 ± 0.58
TBA_{225}	39.2 ± 0.29
$TSST_{L30R/D27A/I46A}$	63.8 ± 0.29
STEBVax	53.5 ± 0.0
$SEA_{L48R/D70R/Y92A}$	38.0 ± 0.0
TSST-1	64.5 ± 0.0
SEB	60.7 ± 0.0
SEA	62.0 ± 0.0

Table 1. Melting temperature of toxoids determined by DSF.

were expressed in *E. coli* and purified by column chromatography. The fusion proteins showed a single peak in SEC-HPLC with an apparent molecular weight of 90.3 kDa (Fig. 5B) and a single band in SDS-PAGE (Fig. 5C) and Western blot (Fig. 5D) analysis. Thermostability of the constructs was evaluated by differential scanning fluorimetry (DSF). $TSST\text{-}1_{L30R/D27A/I46A}$, STEBVax and $SEA_{L48R/D70R/Y92A}$ had melting temperatures of 63.8, 53.5 and 38 °C respectively. We found that TBA and TBA_{225} have an average melting temperature of 41.8 °C and 39.2 °C, respectively indicating that the additionally introduced mutation only had marginal impact on the stability of SEAVax and consequently TBA_{225} (Table 1).

Safety of TBA and TBA_{225} along with individual toxoids was evaluated in PBMCs from four donors. As shown in Fig. 6A–D, neither $SEA_{L48R/D70R/Y92A}$ or SEA_{H225A} were fully attenuated. In contrast, $SEA_{L48R/D70R/Y92A/H225A}$ was completely inactive except in a highly sensitive donor (Fig. 6D) that displayed a low response at high concentration of the toxoid. TBA displayed various levels of residual toxicity in all four donors, while TBA_{225} was fully attenuated, even more than $SEA_{L48R/D70R/Y92A/H225A}$ (Fig. 6D). The data indicate that TBA_{225} mutant represents a safe vaccine candidate.

Immunogenicity of the superantigen fusion toxoids. We next evaluated the immunogenicity of TBA and TBA_{225} in comparison with a cocktail of the three toxoids using Alhydrogel as adjuvant. Groups of 10 Balb/c mice were immunized three times on days 0, 14, and 28 with the toxoid cocktail (6.6µg each), TBA (20 µg), or TBA_{225} (20 µg) or BSA (20µg) as control. Sera were collected on day 35 and used to determine the ELISA binding and toxin neutralization activity (TNA) titers. As shown in Fig. 7A, all three formulations induced high levels of IgG titers against SEA and SEB. Notably, no anti-TSST-1 IgG titer was observed in the group immunized with the toxoid cocktail while both fusion proteins induced high titers. This was likely due to TSST-1's poor adsorption to Alhydrogel under the conditions used for formulation (Supplementary Fig. S1), however, an immunological interference in the mixture cannot be ruled out. Similarly, high levels of TNA titers were observed against SEA and SEB with the cocktail and against all three antigens with the fusion proteins. TBA and TBA_{225} elicited comparable binding and neutralizing titers to both SEA and SEB in comparison to SAg toxoid cocktail and TBA. Consistent with the ELISA data the cocktail failed to elicit neutralizing titers to TSST-1, while TBA and TBA_{225} elicited similar neutralizing titers to TSST-1 (Fig. 7B), indicating that a fusion-protein can compensate for the low immunogenicity of individual TSST-1 toxoid. In addition, TBA_{225} elicited cross-reactive antibodies to SEC-1, SEC-2, SpeC and SEK as well as neutralizing titers to SEC-1, SEC-2, SpeC, SEH and SEE determined at 1:100 and 1:40 serum dilution, respectively (Fig. 7C).

TBA_{225} immunization protects against toxin challenge. We next sought to evaluate the ability of TBA_{225} to provide protection against toxin challenge in the TSS mouse model. Six groups of 10 Balb/c mice were

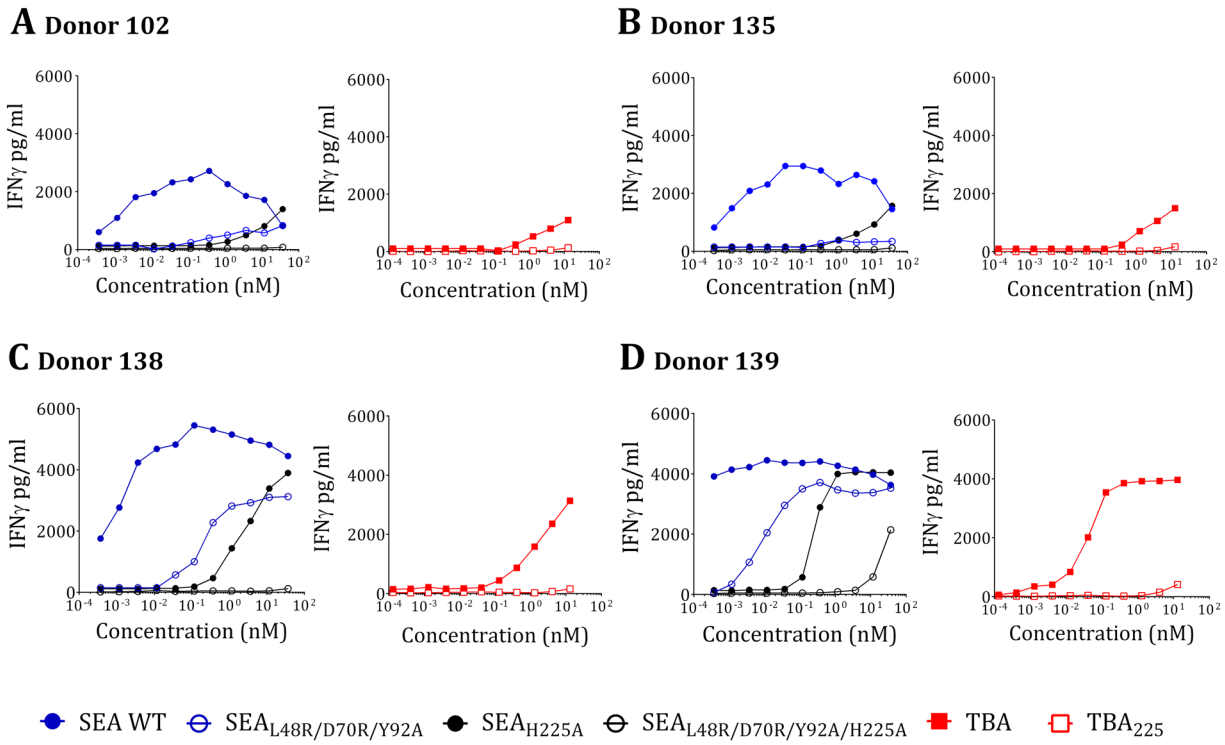

Figure 6. PBMC stimulation (interferon-gamma response) profile to donors (**A**)102, (**B**)135, (**C**)138, (**D**)139 by SEA and its mutants $SEA_{L48R/D70R/Y92A}$, SEA_{H225A}, $SEA_{L48R/D70R/Y92A/H225A}$ as well as TBA and TBA_{225}.

immunized with either BSA as negative control or TBA_{225} as described above. Mice were then challenged with SEA, SEB, or TSST-1 on day 35 followed by LPS. While all BSA immunized mice succumbed to toxic shock within 24 h, TBA_{225} immunization provided 100% protection against SEB and TSST-1 and 90% protection against SEA (Fig. 8A). Analysis of day 35 sera showed that TBA_{225} immunized mice had high binding and neutralizing titers to SEA, SEB and TSST-1 in contrast to BSA immunized mice (Fig. 8B,C).

TBA_{225} vaccine elicits cross-reactive and cross-neutralizing antibodies. To evaluate the breadth of reactivity of antibodies elicited by TBA_{225} we generated polyclonal antisera in rabbits and purified total IgG by protein A chromatography. Polyclonal antibodies raised against another staphylococcal toxin, an attenuated Leukocidin F (LukF) molecule ($LukF_{mut1}$)[36] was used as control. The rabbit polyclonal anti-TBA_{225} IgG showed binding to all SAgs tested (Fig. 9A). Anti-TBA_{225} IgG was also tested in TNA assays against purified TSST-1, SEA, SEB, SEC-1, SEH, SEK, SEE, SED, and the streptococcal SpeC and was shown to neutralize all these toxins with different potencies (Fig. 9B). Highest level of cross-neutralization was evident for SEC-1, and SEH, while IC_{50} values for SEK, SEE, SED, and SEE were above 100 μg/ml (Fig. 9B). As expected $LukF_{mut1}$ polyclonal did not elicit any binding (Fig. 9A) or neutralizing titers (data not shown).

We next examined if the anti-TBA_{225} pAbs can inhibit the stimulation of human PBMCs by culture supernatants of various *S. aureus* clinical strains. PBMCs were stimulated in presence of increasing concentration of anti-TBA_{225} or anti-$LukF_{mut1}$ pAbs with overnight culture supernatants of USA300 (SF8300) (1:4000), USA400 (MW2) (1:16), USA200 (1:12), USA1100 (1:400), MRSA252 (1:4), MRSA COL (1:4000), Newman (1:4000), and MN8 (1:40). After a 48 h incubation IFNγ concentration in the supernatants was measured by quantitative ELISA. As expected different strains induced various levels of IFNγ production. USA400, MN8, COL, and SF8300 exhibited the highest level of IFNγ induction (Fig. 9C), while USA1000 and USA200 showed intermediate levels and MRSA252, USA100, and Newman induced low levels of IFNγ. Anti-TBA_{225} IgG was able to inhibit the stimulatory activity in all these supernatants to various degrees (Fig. 9C) indicating that SAg mediated activities in these supernatants are being neutralized.

Discussion

The objective of the presented work was to evaluate if broad neutralization of most prominent staphylococcal SAgs is possible with a single subunit vaccine. We studied the breadth of neutralizing and cross-neutralizing antibodies elicited by natural exposure to staphylococcal superantigens. Our findings show that human antibodies to SEA, SEB, and TSST-1 can neutralize multiple related SAgs and can provide broader neutralization when combined. Concentration of these antibodies in human plasma appears to be fairly low and their elevation to therapeutically meaningful levels would require immunization. To this end we generated a fusion peptide toxoid vaccine (TBA_{225}) consisting of toxoids for SEA, SEB, and TSST-1 and demonstrated its efficacy against toxin challenge *in vivo* and its ability to elicit broad cross-neutralizing antibodies against a wide range of SAgs. Our findings

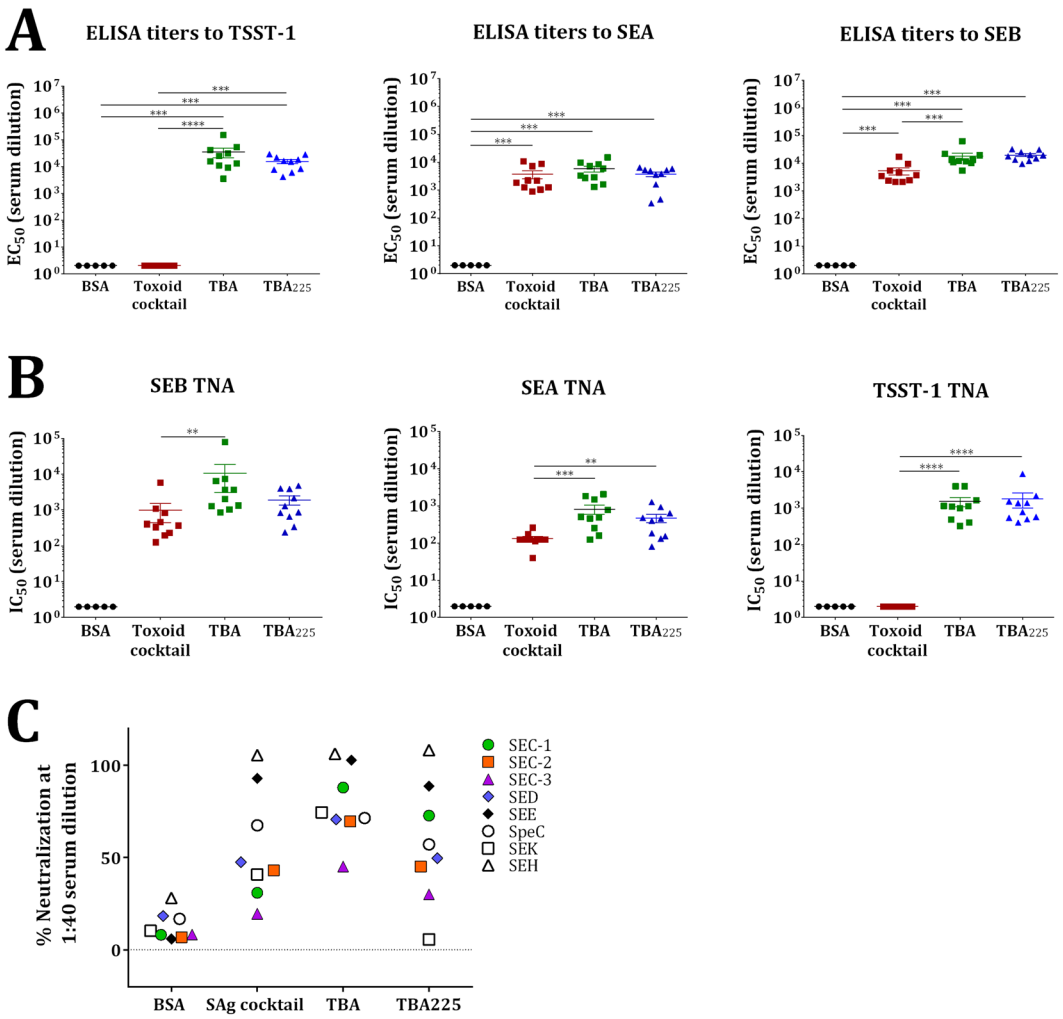

Figure 7. ELISA and TNA titers of mouse sera. Mice were immunized with BSA, toxoid cocktail, TBA or TBA_{225} and serum samples were tested for (**A**) Binding towards SEB, SEA and TSST-1 by ELISA, expressed as EC_{50} values, (**B**) Neutralization of SEB, SEA and TSST-1 determined by inhibition of IFN-γ release by human PBMCs, expressed as IC_{50} values and (**C**) Cross-neutralization of other superantigens expressed as percentage neutralization values at 1:40 serum dilution.

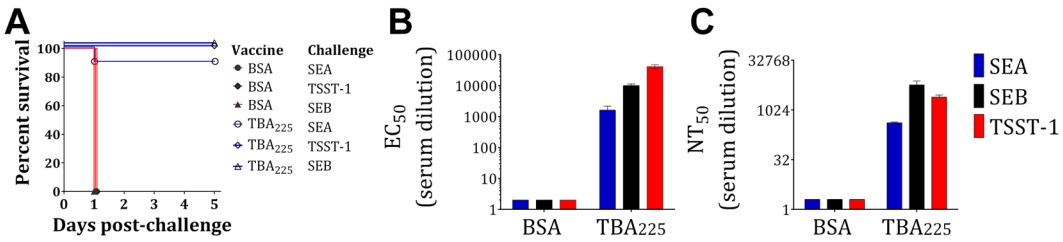

Figure 8. Immunogenicity and efficacy of TBA225. Balb/c mice were immunized with BSA or TBA225, subjected to toxin (SEA, SEB or TSST-1) challenge and monitored for (**A**) survival. Pooled serum samples were tested for (**B**) binding and (**C**) neutralizing titers towards SEA, SEB and TSST-1.

are important for development of a future multivalent toxoid vaccine for *S. aureus* infection and prevention or treatment of superantigen induced diseases and SA infections in general.

Presence of antibodies to SAgs in normal human plasma and IVIG preparations has been previously reported[38–40,55]. However, to our knowledge the anti-SAg antibody content in healthy human plasma has not been quantitatively determined. In a cohort of 30 healthy plasma donors we found that all individuals had detectable levels of antibodies to SEA, SEB, TSST-1, SED, as well as SpeA and SpeC with circulating concentrations ranging from 100 ng/ml to low μg/ml (Fig. 1A). Based on the dose response curve of the affinity purified antibodies (Fig. 2A), full neutralization of SEA, SEB, and TSST-1 requires 50, 300, and 20-fold molar excess of the respective

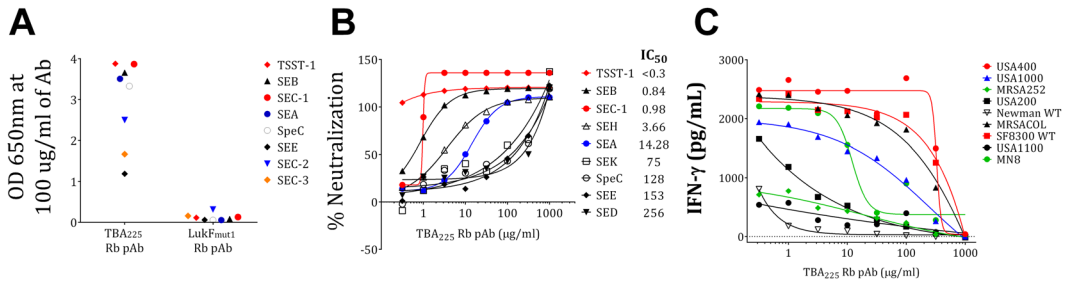

Figure 9. (**A**) Binding, (**B**) Neutralization by TBA_{225} polyclonal antibody to superantigens SEA, SEB, TSST-1, SEC-1, SEC-2, SEC-3, SEH, SEK, SEJ, and SEI, (**C**) Reduction in interferon-gamma production by bacterial supernatants from strains: USA400, USA1000, MRSA252, USA200, Newman WT, MRSACOL, SF8300 WT, USA 1100, and MN8 upon exposure to TBA_{225} rabbit polyclonal.

IVIG-derived polyclonal antibodies. Thus, for example, a concentration of 0.694 μg/ml (4.6 nM) anti-SEB in blood (median of 30 samples, Fig. 1A) can fully detoxify ~0.015 nM of circulating SEB (~0.5 ng/ml). Similarly, the plasma sample in our cohort with the highest anti-SEB titer (4.5 μg/ml, Fig. 1A) and the plasma with the lowest concentration (0.1 μg/ml) would be able to detoxify a maximum blood concentration of ~3.1 and 0.07 ng/ml SEB, respectively. Little is known about the local and systemic levels of SAgs produced during infection. Azuma *et al.* reported circulating SAg concentration in ICU patients in the range of 0.01–0.15 ng/ml[56]. Clearly at least a portion of the population is unable to fully detoxify these levels of circulating toxins. In addition, given that SAgs are produced at the site of infection and then released into the circulation, it is conceivable that the local concentration of SAgs are exponentially higher and far above the neutralizing capacity of circulating natural antibodies as we determined here.

Human immunoglobulin preparations have been used for treatment of streptococcal and staphylococcal TSS with limited success and their utility remains controversial[30–33]. We determined the concentration of anti-SEB antibodies in 28 lots of IVIG and found the median to be 121 μg/g IVIG (range 82–188). Based on this analysis, a dose of 400 mg/kg IVIG would deliver 2.3–5.3 mg of anti-SEB antibodies to a 70 Kg patient yielding added concentrations of 0.5–1 μg/ml specific antibody. Thus, the commonly used doses of IVIG therapy only incrementally increase the circulating concentrations of anti-SAg antibodies.

Our data indicate that vaccination is required to achieve a safe excess of SAg neutralizing antibodies in humans. Anamnestic response to vaccines often leads to exponential increase in antibody titers[57–59]. We recently completed a Phase I clinical trial of a monovalent SAg vaccine and a component of TBA_{225}, STEBVax ($SEB_{L45R/Y89A/Y94A}$)[55]. In this study, a single dose of STEBVax (2.5 μg or greater) led to an average of ~40 fold elevation of anti-SEB titer (range 3.7–194 fold) within 2 weeks[55]. In addition, Schwameis *et al.* tested a recombinant TSST-1 toxoid in healthy human volunteers and demonstrated a strong antibody response to the vaccine[60]. These data indicate that the baseline titers against SAgs can be exponentially elevated by vaccination as opposed to incremental increase achieved by IVIG treatment.

A combination of human polyclonal antibodies against SEA, SEB, and TSST-1 neutralized a wide range of SAgs at levels better or equivalent to the individual antibodies (Fig. 3). Based on this finding we generated a fusion protein consisting of toxoids for these three SAgs, named TBA_{225}, and demonstrated its full attenuation with respect to superantigenic toxicity (Fig. 6). The fusion vaccine elicited homologous and heterologous antibody responses at levels generally higher than a cocktail of the three toxoids, indicating that the fusion not only has simplified the vaccine but also increased its immunogenicity (Fig. 7). TBA_{225} provided full protection against the three superantigens in a toxic shock model (Fig. 8A) and vaccine-elicited antibodies neutralized a wide range of SAgs including SEK, the primary SAg produced by the currently circulating USA300 lineage. TBA_{225}-elicited antibodies also neutralized the intoxication of human PBMCs by crude culture supernatants of multiple clinically relevant *S. aureus* isolates including USA300 (Fig. 9).

To date efforts towards development of vaccines for *S. aureus* have been focused on surface antigens, modeled on the success of capsular polysaccharide vaccines for several bacterial agents. However, all these efforts have failed to achieve the clinical endpoint of preventing staphylococcal infections. At least one of these vaccines, V710, had even a deleterious impact on the outcome of *S. aureus* infection in vaccinated individuals including statistically significant higher multiorgan failure in the vaccine arm (31 vs 17 events; $P = 0.04$) and the observation that the V710 recipients who suffered a *S. aureus* infection were 5 times more likely to die than patients that received the placebo vaccine[6]. A post-hoc study performed on a subset of available sera from these patients suggested that low pre-vaccination IL-2 and IL-17A levels might have predisposed these patients to catastrophic mortality upon *S. aureus* infection[7]. Furthermore, two studies in mice[61] and rabbits[62] also indicate that immunization with surface antigens can induce a detrimental immune response. Collectively, these data indicate that a dysregulated immune response may be the primary cause of lack of response to vaccines or vaccine mediated enhancement of *S. aureus* disease.

Toxoids represent a promising alternative as "anti-virulence" vaccines. This is consistent with reports that lower level of antibodies against toxins correlate with severity of disease[29,63]. Previously, we have reported strong protection against multiple syndromes of *S. aureus* using attenuated vaccines for pore forming toxins alpha hemolysin and leucocidin components[36,64–66]. Beside their prominent role in TSS, SAgs also impact the virulence of SA through induction of a local excessive inflammatory response, immune subversion by inducing apoptosis of T

and B cells[11,12] and modulation of the function of regulatory T cells (Tregs)[13–15], innate lymphoid cells (ILCs)[16], and unconventional T cells such as γδ T cells[17,18], NKT cells[19–21], and mucosa associated invariant T (MAIT) cells[22]. SAgs are also implicated in the pathogenesis of neonatal exanthematous disease, infective endocarditis, sepsis, and atopic dermatitis[23]. Several groups have reported partial protection against *S. aureus* infections in various animal models using vaccines or antibodies against SEB[67], SEA[68], TSST-1[69], SEC.[70] and SEK[71]. SAgs are implicated in sepsis based on higher prevalence in septicemia-causing isolates[72], significantly higher and more frequent detection of SAgs in patients with sepsis[56], and serological studies in bacteremic patients[29] and are known to alter the human immune system. Thus, the superantigen vaccine presented in this report can be a valuable addition to attenuated pore forming toxoids to generate a multivalent toxin-based vaccine for *S. aureus*.

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Author Contributions

M.J.A. conceived the research concept and as principal investigator guided the studies. A.V. and H.K. designed and executed the animal models, *in vitro* experiments and drafted the manuscript along with M.J.A. R.A. and T.K. contributed towards design and production of the vaccine. G.L., S.C., D.D. and L.A. performed human PBMC and plasma experiments. Shweta K. contributed to stability testing. Subramaniam K., Y.O. and F.W.H. facilitated testing of immunogenicity and safety of the vaccine. All authors contributed to manuscript discussion and final stages of writing and review.

Additional Information

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Competing Interests: M.J.A. has stocks in integrated Biotherapeutics, H.K. and F.W.H. have stock options in integrated Biotherapeutics.

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